Supporting Information

TNF-α promotes c-REL/ΔNp63α interaction and TAp73 dissociation from key genes that mediate growth arrest and apoptosis in head and neck cancer

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Supplemental Experimental Procedures

Reagents. The expression plasmids for ΔNp63α and TAp63α were kindly provided by Drs. James W. Rocco and Leif W. Ellisen (Massachusetts General Hospital and Harvard University, Boston, MA) (1). c-REL expression plasmids were kindly provided by Dr. Thomas Gilmore (Boston University, Boston, MA) (2). The luciferase plasmid containing ~2.6kb p21 promoter sequence was from Dr. Bert Vogelstein's laboratory (3). The sequence of p63 siRNA targeting the unique exon in the N-terminal of ΔNp63 was according to previous publication (4), and the siRNA was made by Integrated DNA Technologies (IDT). The siRNA specifically targeting TAp63 was from ON-TARGETplus of SMART selection (Dharmacon). Control siRNAs were purchased from Dharmacon or Qiagen. Recombinant TNF-α was purchased from R&D Systems.

Immunohistochemical analysis of HNSCC tissue specimens. Frozen tissue samples of HNSCC without personal identifying information were obtained from the Cooperative Human Tissue Network (CHTN), and studies were exempted from IRB review by the

NIH Office of Human Subjects Research. Detailed procedures for immunohistochemistry (IHC) and histoscore determination, which combines both staining intensity and percentage cells stained, were described previously (5, 6). The primary antibodies used for immunostaining were rabbit anti-cREL (Cell Signaling 4727), goat anti-ΔNp63 (Santa Cruz 8609), mouse anti-p73 (Imgenex 259A), mouse anti-TP53 (Calbiochem OP43A) , mouse anti-pan cytokeratin (Novocastra). Photomicrographs were taken at 400X and 1000X magnification.

Cell Lines. A panel of 9 HNSCC cell lines from the University of Michigan squamous cell carcinoma (UMSCC) series was obtained from Dr. T.E. Carey (University of Michigan, Ann Arbor, MI). These UMSCC cell lines were previously characterized and found to reflect molecular and phenotypic alterations expressed *in situ* and important in the pathogenicity of HNSCC (7). The *TP53* mutation status of these cell lines was analyzed by bi-directional DNA sequencing of exons 4-9 for mutation (7, 8). UM-SCC cell lines and primary human epidermal keratinocytes (HEKA) were cultured as previously described (9).

Real time RT-PCR. RNA isolation and cDNA synthesis were performed as previously described (10). Real time PCR primers and probes for ΔNp63 and TAp63 were synthesized by Applied Biosystems, according to a previous publication (11). Other primers and probes were purchased through Assays-on-Demand™ from Applied Biosystems. Amplification conditions were: 2 min at 50^{o}C and 10 min at 95^{o}C, followed

by 40 cycles of 15 sec at 95^{o}C and 1 min at 60^{o}C, carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative gene expression values were calculated after normalization to 18S rRNA. The primers to amplify TAp73 (forward, CACGTTTGAGCACCTCTGGA; reverse, GAACTGGGCCATGACAGATG) and ΔNp73 (forward, TGTACGTCGGTGACCCCGCAC; reverse, TCGGTGTTGGAGGGGATGACA) were as described previously (12). Primers for p21, NOXA and PUMA promoters were -2283 p21 (forward, AGCAGGCTGTGGCTCTGATT, reverse, CAAAATAGCCACCAGCCTCTTCT); -1380 PUMA (forward, CCAGATTTGTGGTGAGTGTG, reverse, TCACACCTGTGACAGCTTCT) and -2930 NOXA (forward, ATCGAAATGATCAGATGTGC, reverse, TAGGGAATTCTAACGCTTCA). The cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems), and quantified for 50 cycles by an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The specificity of PCR products was assured by running a 2% agarose E-Gel (Invitrogen). Results were normalized to hypoxanthine phosphoribosyltransferase 1 (Hu-HPRT) expression. Each sample was assayed in triplicate and data were presented as mean ± SD.

Western Blot. Whole cell lysates were obtained using an Extraction Kit from Active Motif according to the manufacturer's protocol (Active Motif). The Western blot analysis was performed using 4-12% Bis-Tris gradient gels (Invitrogen) as previously described (8). Primary antibodies used were rabbit anti-TP53 (Santa Cruz-6243), mouse

anti-p63α (Santa Cruz-8344), mouse anti-TAp73 (Imgenex-246), mouse anti-cREL (Cell Signaling-4727), goat anti-p21 (Santa Cruz-397G), rabbit anti-beta-actin (Cell Signaling-4967). Signal was detected using horseradish peroxidase linked anti-mouse (1:4000), anti-goat (1:4000) or anti-rabbit (1:4000) secondary antibody as appropriate (Chemicon International). Each blot was incubated with Pierce Super Signal West Pico substrate (Pierce Biotechnology) and exposed to Kodak X-OMAT film.

Plasmid and siRNA Transfection. Transfection was performed as previously described (10). Briefly, the sequence of p63 siRNA targeting the unique exon in the N-terminal of ΔNp63 was according to previous publication (4), and the siRNA was made by Integrated DNA Technologies (IDT). The siRNA specifically targeting TAp63 was synthesized by Dharmacon. The luciferase plasmid containing ~2.6kb p21 promoter sequence was from Dr. Bert Vogelstein's laboratory (3). The plasmid DNA or siRNA (Dharmacon or IDT) and Lipofectamine 2000 (Invitrogen) were diluted in Opti-MEM medium (Invitrogen) separately in RT for 5 min, and then mixed and incubated at RT for 20 min to 1 hr. Cultured cells were changed to complete cell growth medium without antibiotics before DNA/lipofectamine mixtures were added. Cells were transfected for 5 hr at 37^{o}C in the CO2 incubator, and the medium was removed and fresh complete growth medium without antibiotics was added.

Reporter gene assays. Reporter gene activities were assayed by a chemi-luminescence detection protocol from the Dual-Light System kit and measurements were obtained using a Wallac VICTOR2 1420 Multilabel Counter. The reporter gene activities were adjusted to

β-gal activities or WST1 reagents (Roche). Each sample was assayed in triplicate and data were presented as mean ± SD.

Coimmunoprecipitation analysis (IP). Lysates and co-immunoprecipitation was performed as previously described (13). In brief, lysates were precleared with appropriate antibody and beads and then incubated overnight at 4°C with primary anti-bodies: rabbit anti-c-REL (Santa Cruz-71), mouse anti-p63α (Santa Cruz 8344), mouse anti-TAp73 (Imgenex 246), rabbit anti-TP53 (Santa Cruz-6243), or control IgG (mouse, Santa Cruz-2025; rabbit, Santa Cruz-2027) . Protein A/G Plus beads (Active Motif) were added for the final hour, and samples were washed four times with RIPA lysis buffer (Upstate Biotechnology). The immunoprecipitants were eluted by directly boiling the beads in SDS sample buffer (Invitrogen, cat. NP0007). 2ug of antibodies for immunoprecipitation were added in 500ul of cell lysates. The antibodies were shown in Table S3.

Electrophoretic mobility shift assay (EMSA). The EMSA was performed using radioactive labeled oligonucleotides. The oligonucleotides for EMSA covering p21 promoter p63-binding site #1 (-2283bp, 5′-TGGCCGTCAGGAACATGTCCCAACATGTTGAGCTCTGGCA-3′, Figure S3C) was [α-^{32}P]-end-labeled with 10 U of T4 polynucleotide kinase (New England Biolabs) according to a previous publication (14): Nuclear extracts (6 ug/reaction) were incubated at room temperature with 1 ul of labeled probe (20,000 cpm) and resolved by gel electrophoresis. For a negative control, unlabeled oligonucleotide was added in 200-fold

excess. Gel analysis was carried out in 4% acrylamide/0.5% TBE gels. Dried gels were placed on a phosphor screen for 24 hours.

Chromatin Immunoprecipitation Analyses (ChIP). ChIP assays were performed using the Magna ChIP™ G kit (Upstate Biotechnology) as previously described (15). Briefly, Cells were crosslinked with 1% formaldehyde. To generate DNA fragments of 0.2–1 kb, 1 x 10^6 cells were sonicated by S-4000 Cup Horn (Misonix, Inc) on 50% power for 4 min with an on–off interval of 20 second. Chromatin fragments were incubated with the antibody overnight and collected in protein-G agarose (Upstate Biotechnology). After cross-linked products were reversed, the supernatants were treated with proteinase K for 3 h at 65 °C, followed by phenol/chloroform purification and ethanol precipitation. ChIP samples were then analyzed by standard qPCR reaction using the following primers: Fwd, AGCAGGCTGTGGCTCTGATT; Rev, CAAAATAGCCACCAGCCTCTTCT. The primers used to amplify p21 promoter were previously described (16).

Cell Proliferation Assay. Cell proliferation assay was performed using the WST-1 cell proliferation reagent (Roche) as previously described (17). In brief, cells were plated in 96-well plates at a density of 3000 cells per well in 100ul medium, cultured overnight, and treated according to the applied protocol. After treatment, WST-1 cell proliferation reagent was added and samples were incubated at 37°C for 1–2 hours and absorbance measured at 490 nm.

DNA based cell cycle and apoptotic flow analysis. UM-SCC 11A cells were plated in 6 well plates and transfected as previously (8). At 48, 72 or 96 hours, cells were trypsinized, counted and labeled using the Cycletest Plus DNA regent kit (BD Biosciences) following the standard protocol. Samples were run on FACS Canto machine within 1 hour and analyzed using DIVA flow cytometric analysis software.

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Supporting Information Figure Legends

Figure S1. Expression of TP53, p63, p73 and c-REL in UM-SCC cell lines.

A, Basal protein expression of c-REL, ΔNp63, p73, and TP53 were analyzed by Western blot in whole cell lysates, nuclear or cytoplasmic fractions of HEKA and nine UM-SCC cell lines. As controls for loading, β-actin was used for whole cell lysates and the cytoplasmic fraction, and OCT-1 was used for the nuclear fraction. Basal mRNA levels for B, TP53, C, ΔNp63 and TAp63, D, ΔNp73 and TAp73, and E, c-REL, were detected by qRT-PCR in HEKA and nine UM-SCC cell lines, including 4 lines with deficient expression of wt TP53 (UM-SCC 1, 6, 9), and five lines with mtTP53 (UM-SCC 11A, 11B, 22A, 22B, 38 and 46). F, p63α proteins were detected in whole cell lysates and compared with positive control using cell lysates expressing human TAp63α, ΔNp63α, TAp63γ proteins.

Figure S2. ChIP assays of c-REL, ΔNp63a and TAp73 binding A, p63 binding sites on the *p21WAF1*, *PUMA* and *NOXA* promoters (~3000 bp upstream from Transcription Start Site, TSS) were predicted by using Genomatix Matrix position weighted matrices. ▼indicates the binding site amplified in the q-PCR ChIP assay experiments. B-E, ChIP assays were performed using specific antibodies to detect protein binding activities to promoter sites as indicated. B, Basal binding activities of c-REL, ΔNp63α, and TAp73, but not REL-A (p65) were detected on the binding site of *p21WAF1* promoter in UM-SCC 46. C, Increased c-REL, and decreased TAp73 binding with TNF (20ng/ml) treatment in UM-SCC 46. D, following transfection by c-REL expression vector in UM-SCC 1 for 48h. E, decreased c-REL, ΔNp63α and increased TAp73 following

transfection by c-REL siRNA in UM-SCC 22A.

Figure S3 Expression of p21WAF1 in UM-SCC cell lines; effects of TNF-α, c-REL and TAp73 siRNA or expression vector transfection on p21WAF1 expression

A, mRNA and B, protein expression of *p21WAF1* in HEKA and nine UM-SCC cell lines was detected by qRT-PCR and Western blot analysis. HEKA cells undergoing contact inhibited growth arrest near confluence were used as a positive control. Lower levels of *p21WAF1* mRNA and protein were observed in cell lines with deficient wtTP53 expression (1, 6, 9, 11A). C, Nuclear c-REL translocation and decreased *p21WAF1* protein expression following TNF-α treatment. Cytoplasmic fraction (CF) and Nuclear fraction (NF) extracts from TNF-α treated UM-SCC 22A cells were analysed by anti-c-REL and anti-p21 Western blotting. β-actin and OCT-1 were used as controls for loading and fractionation. D, *p21WAF1* expression by quantitative RT-PCR after transfection of UM-SCC 22A by c-REL siRNA or both c-REL and TAp73 siRNA; or E, following transfection of UM-SCC 1 by c-REL or both c-REL and TAp73 expression vector.

Figure S4. Modulating c-REL differentially affects cellular proliferation, and apoptosis in UM-SCC22A cells expressing increased ΔNp63 and TAp73 with mtTP53. A, Phase contrast photomicrographs of UM-SCC 1 and 22A cell cultures 48h after knockdown of c-REL. The cell density of UM-SCC 22A, but not UM-SCC 1, was inhibited after c-REL was knockdown. Flow cytometric analysis of sub-G0 DNA content after B, c-REL siRNA, or C, overexpression of c-REL plasmid, reveals marked effects on

sub-G0 DNA fragmentation of UM-SCC 22A when compared with UM-SCC1 cells.

Figure S5. Expression of TP53, p63, p73 and c-REL in HNSCC tissue specimens

A, Immunohistochemical detection of c-REL, ΔNp63, p73, and TP53 in representative matched squamous mucosa and HNSCC tumor specimens with higher magnification (100x). Pancytokeratin staining was used as a positive control to highlight epithelia. B, Immunostaining intensity was quantified in 24 HNSCC specimens by histoscore: 0/+, no or minimal staining; ++, intermediate staining; and +++, strong staining. Percentages of tumor specimens in each score category are presented.